# MODIFICATION OF CARBOXYL GROUPS IN THE BINDING SITE OF TRYPSIN WITH THE MEERWEIN REAGENT*1

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Summary Modification of the carboxyl group in trypsin by the Meerwein reagent procedure developed in our laboratories has shown that 1.6 to 1.7 carboxyl groups can be alkylated with significant loss ($\sim$80%) of the BAEE activity of the enzyme. The presence of a competitive inhibitor, β-naphthamidine, strongly protects the activity. The modified enzymes retained much activity for a nonspecfic substrate. These results are interpreted in terms of preferential alkylation of the carboxyl group(s) in the binding site of trypsin with the reagent.

A recent paper by Eyl and Inagami (2) of the active site of trypsin prompted us to report the results obtained in our laboratory on chemical modification of carboxyl groups of trypsin.

Kinetic studies of competitive inhibitors have suggested the participation of a carboxyl group in the binding site (Inagami *et al.* (3), and Shaw *et al.* (4)), while the binding of certain inhibitors was found to be dependent on a group with a $pK_a$ of 4.1-4.7 by means of spectroscopic (5) and gel filtration techniques(Tanizawa *et al.*(6)). Recently Shaw *et al.* reported the modification of "essential carboxylic acid" side chain of trypsin (7). Since Yonemitsu, Hamada and one of the authors (Y.K.) had developed a mild method for the chemical modification of carboxyl groups of peptides in aqueous solution (8), the use of triethyloxonium fluoroborate $Et_3O^+BF^-_4$ (Meerwein regent;

*1 Chemical Modification of Proteins. Part III. For Part I and II see ref. (1a) and (1b), respectively.

MR) for the alkylating modification of carboxyl group(s) at the binding site of trypsin was undertaken.*2 For comparison, the well-known amidation method by Koshland et al. (10,11) was also examined. In these experiments, β-naphthamidine (β-NA), a good competitive inhibitor (Tanizawa et al. (12)), was employed to protect the carboxyl group(s) in the binding site from modification.

Materials and Methods Trypsin (lot TRL-8GA, 9JB) was purchased from Worthington Biochem.Co. B-Naphthamidine (6) and MR (8,13) were prepared according to the procedure described in literatures. $^{14}$C-(U)-glycine ethyl ester·HCl was prepared from $^{14}$C-(U)-glycine (Daichi Kagaku Yakuhin Co.) as usual. 1-Ethyl-3-dimethylaminopropyl carbodiimide (EDC) was purchased from Otto Chem. Co. Experimental details of the modification reactions are given in the legends to Table I and II. Enzymatic activites were determined with a pH-stat using N-benzoyl-L-arginine ethyl ester-HCl (BAEE) and N-benzoylglycine ethyl ester-HCl (BGEE) as substrates.

Results and Discussion The reaction of trypsin with MR at concentrations of 0.1 and 0.2M resulted in esterification of 1.6 and 1.7 carboxyl groups and concomitant loss of the BAEE activity down to 52.5 and 21.4%, respectively (Table I). In experiments on a model level MR reacts also with histidine and methionine residues (8). Under these conditions, however, the amino acid analysis indicated that the ethylation of these residues in trypsin had not occurred. This observation that the enzymatic activity is sensitive to such a limited modification of carboxyl groups suggests that rather specific modification of the carboxyl group(s) in the anionic binding site of trypsin has taken place. In order to see whether the modified carboxyl groups are essential to the binding site or not, the reaction was carried out in the presence of β-NA. Extent of modification slighty decreased but was relatively insensitive to the presence of the inhibitor. However, more than 70% of the BAEE

*2 In an independent work, Raftery et al. employed MR for alkylation of lysozyme (9).

activity still remained showing clear protective effect of β-NA for the carboxyl group(s) concerned (Table I). For comparison chymotrypsin was simi-

TABLE I Modification of carboxyl groups of trypsin with MR in the presence and absence of β-naphthamidine

| β-NA | MR | Carboxyl esters | Relative enzymatic activity BAEE | BGEE |
|---|---|---|---|---|
| 0 mM | 0.1 M | 1.6 | 52.5% | 86.8% |
| 50 | 0.1 | 1.3 | 70.0 | 89.7 |
| 0 | 0.2 | 1.7 | 21.4 | 38.0 |
| 50 | 0.2 | 1.0 | 74.5 | 98.5 |

Trypsin (10 mg/ml) was dissolved in distilled water, the pH adjusted to 4.5 and MR dissolved in acetonitrile was added dropwise to a concentration of 0.1 M or 0.2M. pH was maintained by a pH-stat with 5N NaOH. Reaction was completed in 1 hr and a cloudy solution resulted. After centrifugation the product was dialyzed exhaustively against $10^{-3}$M HCl and lyophilized. Numbers of esterified carboxyls were determined by coupling the remaining carboxyls of enzyme with $^{14}C(U)$-glycine ethyl ester by means of EDC in 8M urea solution (13). The difference of the numbers of coupled $^{14}C(U)$-glycine residues (estimated by radioactivity incorporated) between modified and unmodified trypsin under the same conditions gives the numbers of carboxyl esters.

TABLE II Modification of trypsin with EDC and GEE

| β-NA | Moles of GEE incorporated per mole of trypsin | Relative enzymatic activity BAEE | BGEE |
|---|---|---|---|
| 0 mM | 13.6 | 10.8% | 20.3% |
| 50 | 12.2 | 40.5 | 36.7 |

Trypsin (10 mg/ml) and $^{14}C(U)$-glycine ethyl ester were dissolved in distilled water, the pH adjusted to 4.75, and the reaction initiated by addition of EDC to a concentration of 0.25M. The same amount of EDC was additionally supplied after 3 hrs. pH was maintained at 4.75 with 1 N HCl on a pH-stat. Reaction was terminated by adjusting the pH to 3.0 with HCl after 6 hrs and the reaction mixture was dialyzed exhaustively against $10^{-3}$M HCl at 4°. Redioactivity incorporated was determined by scintillation counting.

larly treated with MR in the absence of β-NA to modify 2.6 carboxyl groups. This ethylated chymotrypsin retained 80% of the activity as measured with acetyl-L-tyrosine ethyl ester as a substrate. Apparently ethylation of carboxyl groups not relevant to the binding site of chymotrypsin had no significant influence on the activity in contrast to the case of trypsin.

An alternate method for modification of carboxyl groups was also examined using EDC and glycine ethyl ester (GEE). The results are briefly presented in Table II. The data are substantially in agreement with those reported by Inagami et al. (2) except that an ester was employed instead of an amide, more excess of the reagents were used and therefore more carboxyl groups (12-13 residues) were modified. It must be noted that about 40% of the BAEE activity remained after 12 carboxyls had been modified in the presence of β-NA.

Further, behavior of the modified trypsin to a typical nonspecific substrate (3b), BGEE, was compared with that to BAEE in the hope to confirm the effect of the modification on enzymatic function. In the reaction with EDC and GEE, where rather exhaustive amidation of carboxyl groups had occurred, difference in the effect of modification on the enzymatic hydrolysis of BAEE and BGEE was less pronounced (Table II). However, the results in Table I indicate a distinctly greater reactivity of the MR-modified enzyme with BGEE than with BAEE. This difference may also be interpreted in terms of preferential alkylation of the carboxyl group(s) essential to the binding of substrate by means of MR. In view of the results of Eyl and Inagami's work (2), it is expected that this particular carboxyl groups involve $Asp._{177}$ However, the products obtained in the present work were mixtures and the fractionation of the products is now in progress.

Positively charged alkylating species of MR is probably active-site directed and therefore reaction with carboxylate(s) in the anionic binding site is favored. MR is susceptible to hydrolysis having a half-life time of ca. 10 min., and the competing hydrolysis in aqueous media prevents extensive alkylation even with excess of the reagent. Water soluble carbodiimide has a

much longer half-life time of the order of hour (14), and the carbodiimide-nucleophile system by Koshland et al. (10,11), being nonionic species, seems more suitable for the exhaustive modification of the enzyme rather than for the preferential reaction.

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